

### **REMARKS**

This Reply is responsive to the Office Action dated December 2, 2001. Entry of the foregoing and reconsideration on the merits pursuant to 37 CFR 1.112 is respectfully requested.

The application has been amended as set forth above. In accordance for the new rules for amending applications set forth in 37 CFR 1.121, which took effect on March 1, 2001, a marked up version of the claims showing all amendments is attached hereto as an appendix.

#### **Amendment of the Claims:**

Claims 1, 3, 9, 12-14, 18, 19, 21-23, and 25-30 are amended, responsive to the Office Action mailed December 2, 2001.

Support for the amendment of claims 13, 18, 19, and 21-23 is found on pages 20-21 and original claim 19, which describe transfecting the EG cells with a nucleic acid sequence encoding polypeptides such as enzymes and growth factors, under regulatory control of promoters that direct expression of the protein in eggs, or for recovery from the systemic circulatory system, body fluids, or tissues of an avian having the nucleic acid sequence.

No new matter was added by the amendments.

#### **Regarding Rejection of the Claims Under 35 U.S.C. §112, First Paragraph:**

The claims have been amended to remove the elements or limitations that were the grounds for rejection under 35 U.S.C. §112, first paragraph, set forth in the Office Action. The Applicants greatly appreciate the Examiner's indication of the scope of claims that he regards as being enabled by the description.

Applicants respectfully traverse the rejection of claims directed to making transgenic avian EG cells under 35 U.S.C. §112, first paragraph. As the attached article by Vick et al.

(1993) shows, methods were available for introducing stable genetic alterations into the genomes of cultured avian PGC and EG cells at the time the application was filed.

Reconsideration and withdrawal of the rejections under §112, first paragraph is respectfully requested.

**Regarding Rejection of the Claims Under 35 U.S.C. §112, First Paragraph:**

The claims have been amended to remove the language that provided the grounds for rejection under 35 U.S.C. §112, second paragraph, set forth in the Office Action.

Reconsideration and withdrawal of the rejections under §112, second paragraph is respectfully requested.

**Regarding Rejection of the Claims Under 35 U.S.C. §102:**

Product claims 21 and 22 rejected under 35 U.S.C. §102(b) and 102(e) are canceled.

**Regarding Rejection of the Claims Under 35 U.S.C. §102 and 103 in view of Pain et al., alone or with Simkiss:**

Applicants respectfully direct the Examiner's attention to page 2345, right column, of Pain et al., which states that the long-term cultures were carried out on feeder cells. The present claims are directed to methods that do not use feeder cells.

Reconsideration and withdrawal of the rejections under 35 USC 102 and 103 is respectfully requested.

Applicants acknowledge the obvious-type double patenting rejections set forth in the Office Action, and request that they be held in abeyance until the indication of allowable subject matter.

The issues raised by the Office Action dated December 2, 2001, have been addressed in this Reply, and the claims are now believed to be in form for allowance. If the Examiner has any further questions or issues to raise regarding the subject application, it is respectfully requested that he contact the undersigned so that such issues may be addressed expeditiously.

Respectfully submitted,

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**APPENDIX**

Claims 1, 3, 9, 12-14, 18, 19, 21-23, and 25-30 are amended as follows:

1. (Thrice Amended) A [culturing] method of obtaining [which provides for the production of] avian [PGC and] embryonic germ (EG) cells comprising [the following steps]:
  - (i) isolating primordial germ cells (PGCs) from [a desired] an avian embryo; and
  - (ii) culturing said PGCs in the absence of a feeder layer in a culture medium [containing at least the following growth factors in amounts sufficient to maintain said PGCs for prolonged periods in tissue culture:] comprising
    - (1) leukemia inhibitory factor (LIF),
    - (2) basic fibroblast growth factor (bFGF),
    - (3) stem cell factor (SCF) and
    - (4) insulin-like growth factor (IGF),[for a time period sufficient to produce a culture having a compact multilayer like appearance whereby said culturing is performed in the absence of a feeder layer] so that avian EG cells are obtained [and]  
[(iii) identifying EG cells contained therein].
3. (Amended) The method of Claim 2, wherein the maximal amounts of said growth factors range from about two times to one hundred times said [minimum] minimal amounts.

9. (Amended) The method of Claim 1, wherein the avian EG cells [are identified based on their expression of] produce mouse-stage specific antigen 1, and/or [reactivity] react with EMA-1 or MC-480 monoclonal antibody.

12. (Amended) The method of Claim 1, which further comprises:  
[(iv)] (iii) transfecting or transforming the resultant EG cells with a [desired] nucleic acid sequence.

13. (Amended) The method of Claim 12, wherein said nucleic acid sequence encodes a [therapeutic] polypeptide and is expressed in an egg.

14. (Thrice Amended) A [An improved] method of producing chimeric avians [which comprises] comprising:

- (i) isolating primordial germ cells (PGCs) from an avian;
- (ii) [maintaining such] culturing the PGCs in the absence of a feeder layer in a tissue culture medium containing at least the following growth factors;

- (1) leukemia inhibitory factor (LIF),
- (2) basic fibroblast growth factor (bFGF),
- (3) stem cell factor (SCF) and
- (3) insulin-like growth factor (IGF)

for a sufficient time to produce embryonic germ (EG) cells [whereby said culture is maintained in the absence of a feeder layer];

- (iii) transferring said EG cells into a recipient avian embryo; and

(iv) [selecting for chimeric avians which have the desired EG phenotype] obtaining a germline and somatic cell chimeric avian.

18. (Amended) The method [according to] of Claim 17, wherein said nucleic acid sequence encodes a [therapeutic] polypeptide and is expressed in an egg of an avian embryo having said nucleic acid sequence.

19. (Amended) The method [according to Claim 18, which further includes purifying said therapeutic polypeptide from the eggs of the chimeric avians produced according to step (iv), or the systemic circulating system or body fluids or tissues] of Claim 17, wherein said nucleic acid encodes a polypeptide that can be recovered from the systemic circulatory system, body fluids, or tissues of an avian having said nucleic acid sequence.

21. (Amended) [An avian EG cell line obtained by the culturing method of Claim 1] The method of Claim 17, wherein said nucleic acid encodes a polypeptide that is a growth factor or an enzyme.

22. (Amended) [The cell line of Claim 21, which is a chicken or turkey EG cell line] The method of Claim 12, wherein said nucleic acid encodes a polypeptide that can be recovered from the systemic circulatory system, body fluids, or tissues of an avian having said nucleic acid sequence.

23. (Amended) [The cell line of Claim 21, which contains an inserted nucleic acid sequence] The method of Claim 1, wherein said nucleic acid encodes a polypeptide that is a growth factor or an enzyme.

25. (Amended) A [An improved] method of producing germline chimeric avians [which comprises] comprising:

- (i) isolating primordial germ cells (PGCs) from a Stage XII-XIV avian embryo;
- (ii) maintaining such PGCs in a tissue culture medium containing at least the following growth factors[;]:
  - (1) leukemia inhibitory factor (LIF),
  - (2) basic fibroblast growth factor (bFGF),
  - (3) stem cell factor (SCF) and
  - (4) insulin-like growth factor (IGF);
- (iii) transferring said PGCs into a Stage XII-XIV recipient avian embryo; and
- (iv) [selecting for] obtaining germline chimeric avians [which have the desired PGC phenotype] having germline cells that have the genotype of said PGCs.

26. A [An improved] method of producing germline or somatic cell chimeric avians which comprises:

- (i) isolating primordial germ cells (PGCs) from a Stage XII-XIV avian embryo;
  - (ii) maintaining such PGCs in a tissue culture medium containing at least the following growth factors[;]:
    - (1) leukemia inhibitory factor (LIF),
    - (2) basic fibroblast growth factor (bFGF),
    - (5) stem cell factor (SCF) and
    - (6) insulin-like growth factor (IGF),
- for a sufficient time to produce embryonic germ (EG) cells;

- (iii) [identifying and isolating embryonic germ (EG) cells from said cultured population of primordial germ cells;
- (iv)] transferring said [isolated] EGs into a recipient Stage X avian embryo of the same species as the avian used to obtain said isolated, purified PGCs;
- [(v)] (iv) allowing said recipient avian to develop into a [bird] germline or somatic cell chimeric avian having germline or somatic cells that have the genotype of said PGCs]; and
- (vi) selecting for germline or somatic cell chimeric avians that express the PGC phenotype.]

27. (Amended) A method for producing avian embryonic germ (EG) cells comprising[ the following steps]:

- (i) isolating a [pure] population of primordial germ cells (PGCs) from a Stage XII-XIV avian embryo;
- (ii) culturing said [pure] population of PGCs for a period of at least fourteen days in tissue culture in the absence of a feeder layer [sufficient to produce a culture having a compact multilayer like appearance]in a culture medium comprising:
  - (1) leukemia inhibitory factor (LIF),
  - (2) basic fibroblast growth factor (bFGF),
  - (3) stem cell factor (SCF) and
  - (4) insulin-like growth factor (IGF)]; and]
  - [(iii) identifying the EG cells contained therein] so that avian EG cells are produced.

28. A method for producing a chimeric avian[s] comprising:

- (i) isolating a [pure] population of primordial germ cells (PGCs) from a Stage XII-XIV avian embryo;
- (ii) culturing said [pure] population of PGCs for a period of at least fourteen days in tissue culture[;] in the absence of a feeder layer in a culture medium comprising:
  - (1) leukemia inhibitory factor (LIF),
  - (2) basic fibroblast growth factor (bFGF),
  - (3) stem cell factor (SCF) and
  - (4) insulin-like growth factor (IGF);
- (iii) transferring said [purified] PGCs into a recipient avian embryo of the same species as the avian used to obtain said isolated [, purified] PGCs;
- (iv) allowing said recipient avian embryo to develop into a chimeric avian [bird; and
- (v) selecting for chimeric avians that express the PGC phenotype].

29. (Amended) A method for producing a germline chimeric avian[s]  
comprising:

- (i) isolating a [pure] population of primordial germ cells (PGCs) from a Stage XII-XIV avian embryo;
- (ii) culturing said [pure] population of PGCs for a period of at least fourteen days in tissue culture[;] in the absence of a feeder layer in a culture medium comprising:
  - (1) leukemia inhibitory factor (LIF),
  - (2) basic fibroblast growth factor (bFGF),

- (3) stem cell factor (SCF) and
- (4) insulin-like growth factor (IGF);
- (iii) transferring said purified PGCs into a recipient Stage XII-XIV avian embryo of the same species as the avian used to obtain said isolated, purified PGCs;  
and
- (iv) allowing said recipient avian embryo to develop into a germline chimeric avian [bird; and
- (v) selecting for germline chimeric avians that express the PGC phenotype].

30. (Amended) A method for producing germline or somatic cell chimeric avians comprising:

- (i) isolating a [pure] population of primordial germ cells (PGCs) from a Stage XII-XIV avian embryo;
- (ii) culturing said [pure] population of PGCs for a period of at least fourteen days in tissue culture[;] in the absence of a feeder layer in a culture medium comprising:
  - (1) leukemia inhibitory factor (LIF),
  - (2) basic fibroblast growth factor (bFGF),
  - (3) stem cell factor (SCF) and
  - (4) insulin-like growth factor (IGF);
- (iii) [identifying and isolating embryonic germ (EG) cells from said cultured population of primordial germ cells;]
- [(iv)] transferring said [isolated] EGs into a recipient Stage X avian embryo of the same species as the avian used to obtain said isolated[, purified] PGCs; and

[(v)] (iv) allowing said recipient avian embryo to develop into a [bird] germline or somatic cell chimeric avian[: and

(v) selecting for germline or somatic cell chimeric avians that express the PGC phenotype].



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# Transgenic birds from transformed primordial germ cells

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[Plate 1]

## SUMMARY

Primordial germ cells (PGCs) are the progenitor cells for the gametes. They can be obtained from avian embryos by dissociating the germinal crescent region, where they accumulate, or by sampling the blood at the time of their migration to the gonad. PGCs were obtained from these sources and transfected with defective retroviruses. These manipulated cells were injected into recipient embryos to form chimaeras which grew to sexual maturity and produced offspring, some of which contained the foreign DNA. This is the first example of the direct use of PGCs to produce transgenic offspring.

## 1. INTRODUCTION

Several attempts have been made to introduce foreign DNA into the avian genome to form a transgenic bird. These have included coating the sperm with DNA before fertilization (Gavora *et al.* 1991), attempting microinjection into the male pronucleus of the fertilized egg (Perry *et al.* 1991), and injecting replication-competent (Salter *et al.* 1986) or replication-defective (Bosselman *et al.* 1990) retroviruses into the blastoderm. At the present time, only the infection of the blastoderm by retroviruses has been successful in introducing exogenous genes into the chicken germ line. There are several reasons for these difficulties but the most obvious relate to the avian system of reproduction in which the presence of a large yolky egg with many supernumerary pronuclei (Eyal-Giladi & Kochav 1976) and the fact that the embryo develops to a stage of about 50 000 cells before it is laid (Spratt & Haas 1960) make the standard techniques difficult to apply. To overcome these constraints attention has shifted towards the possibility of producing chimaeric animals as vehicles for gaining access to the avian genome. The most obvious way of doing this is to use primordial germ cells (PGCs) because these are the progenitors of the gametes. Thus, if these cells could be isolated, transformed with foreign DNA and introduced into recipient embryos, some of them should populate the gonads to produce a chimaeric organ. Gametes derived from such cells would produce transgenic animals in the subsequent generation.

In previous work we have shown that it is possible to transfer PGCs from one chick embryo to another to form animals with such chimaeric gonads (Simkiss *et al.* 1989). Subsequently we demonstrated that it was

possible to transfect these donor PGCs with a defective retrovirus so that the foreign DNA was incorporated into the gametes (Simkiss *et al.* 1990). In both these experiments we took advantage of two features of avian development that are unusual among vertebrates. In birds, and a few reptiles, the primordial germ cells accumulate very early in development at an extra-embryonic site in front of the head at the junction of the area pellucida and the area opaca. In the domestic fowl this so-called germinal crescent forms after 2 d of incubation. After about 2½ d of incubation these cells show a second unusual feature in that they migrate via the developing blood system, and are transported to the germinal ridge where they settle in what is to become the definitive gonad (Nieuwkoop & Satawrya 1979). Primordial germ cells can, therefore, be obtained either directly from the germinal crescent region or indirectly by taking carefully timed blood samples from the stage 16 embryo (Al Thani & Simkiss 1991). These cells can then be introduced into recipient embryos where they contribute to the formation of a chimaeric gonad.

The present work was undertaken to quantify some of these processes and to establish that it is possible to use PGCs to produce transgenic birds.

## 2. MATERIALS AND METHODS

A retrovirus-free strain (line zero) of White Leghorn fowl (Astrin *et al.* 1979) was used in most of these experiments. When other birds were required an in-bred line of Rhode Island Reds was used.

Fertile eggs were incubated at 37.5 °C and 70% relative humidity in a forced air incubator (Brinsea Ltd). Embryos were exposed by removing a small piece of shell, and their stage of development was determined from the morphological criteria of Hamburger & Hamilton (1951). Germinal cres-

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cents were obtained from embryos at stage 11 (roughly 40 h incubation) and blood samples were taken from embryos at stage 15 (roughly 55 h incubation). Germinal crescents were dissociated in No-zyme (JRH Biosciences, Sera-Lab Ltd) for 15 min, washed in Hanks balanced salt solution and dispersed in a syringe. The cell suspension was centrifuged at 1000 r.p.m. (Eppendorf 5402) for 30 s and any yolk cells and debris were removed. Primordial germ cells were resuspended in 200 µl Hanks saline, and a 5 µl sample was spun onto a slide (Cytospin 3 Shandon Scientific Ltd). Blood samples were taken from the dorsal aorta of suitable embryos by using a fine needle and syringe. A 5 µl sample was smeared onto a slide for cell counting. The viability of cell preparations was determined using erythrosin B (5 g l<sup>-1</sup>) for 2 min as a stain exclusion test.

Two defective retroviruses were used to transform PGCs. The construction of a defective spleen necrosis virus (SNO21) has been described by Meyers *et al.* (1991). Some clones of this retrovirus showed extensive reorganization in the helper cell line, and a second vector based on a defective avian leukosis virus (NLB) was therefore used in later work. The construction of this retrovirus is given in detail by Cosset *et al.* (1991). It consists of the long terminal repeat (LTR) sequences of the Rous associated virus (RAV-2) with *neo* & *lac Z* genes inserted between the *gag* initiator codon and the 3' end of the *env* gene (figure 2). The defective retrovirus was screened periodically to ensure that no replication-competent virions were produced. Transfection of cells was done using equal volumes of cell suspension and replication defective retrovirus, containing roughly 10<sup>6</sup> virions cm<sup>-3</sup> which were mixed on a rotating platform for 20 min at 37 °C. Transfected cells were washed and 5 µl samples injected into the vasculature of stage 15 embryos by using glass micropipettes produced with a Narishige micropipette puller and microforge. The window in the shell was covered with Micropore surgical tape (3M Health-care) and the eggs were subsequently incubated normally. Viability was checked by routine candling and selected hatchlings were raised to sexual maturity.

In the NLB experiments, line zero (White Leghorn) PGCs were transfected with the defective retrovirus, and these cells were then introduced into Rhode Island Red recipient embryos. Hatchlings were raised to sexual maturity and crossed with White Leghorns.

DNA was prepared from tissue samples by homogenizing and digesting them with proteinase K and RNase before phenol-chloroform extraction and ethanol precipitation. Samples of DNA (0.1–0.5 µg) were digested with restriction endonucleases at 37 °C for 6–8 h and run on electrophoresis gels with *Hind* III digested bacteriophage λ as molecular mass markers. Southern blots were prepared and DNA identified by using [<sup>32</sup>P]-labelled probes produced by nick translation of the original plasmids (Maniatis *et al.* 1982). In the case of SNO21 this screening was based upon detecting a 6.7 kilobase fragment of DNA that hybridized to the [<sup>32</sup>P] probe. This fragment was predicted from the original map of the vector (Meyers *et al.* 1991) which contained two *Sst* I cutting sites at this position, and this was confirmed by transfecting quail fibroblasts (QT 35 line) with vSNO21 and probing the DNA. The NLB vector contains two unique *Bam* HI cutting sites 4.5 kilobase pairs apart and this fragment was identified by hybridizing to the equivalent [<sup>32</sup>P]-labelled plasmid.

### 3. RESULTS

The primordial germ cells of the fowl embryo are typically 15–20 µm in diameter with a large eccentric

nucleus (8 µm diameter) and numerous yolk and glycogen granules in the cytoplasm (Simkiss 1991). The mean number of PGCs per germinal crescent of a stage 11 line 0 White Leghorn embryo was 95 ± 8, and the viability of the PGCs decreased to roughly 68% during their isolation from the germinal crescent. This was attributed to damage during their extraction (table 1). PGCs from the blood averaged 11 ± 3 per 10 µl sample.

Opening eggs incubated for 2 d resulted in roughly 20% mortality of embryos and intravascular injection caused a further 40% mortality (figure 1). To test whether donor PGCs had been incorporated into their gonads, embryos were sampled at 18 d, as this corresponds with the maximum number of gonocytes.

DNA derived from the vSNO21-transfected cells was

Table 1. *Viability of PGCs derived from germinal crescents*

stage of procedure	viability (%)
treatment with No-zyme	93
tissue dissociation in syringe	84
centrifugation and resuspension	68
transfection with vector	54

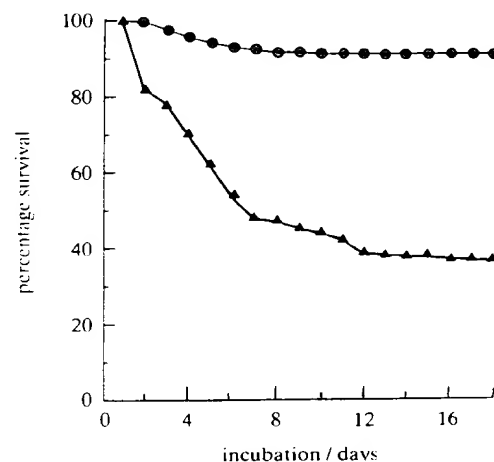


Figure 1. Percentage survival of control (circles) and recipient (triangles) embryos after being injected with transformed PGCs ( $n = 200$  for each data set).

Table 2. *Effect of source of PGCs on chimera production in 18 d embryos by using SNO21*

source	number of embryos	number with positive DNA
blood	69	2 (3%)
germinal crescent	22	5 (23%)

Table 3. *Number of offspring analysed before a transgenic chick was obtained*

bird	retrovirus	number of embryos	number transgenic
cock 1	SNO21	56	1 (2%)
cock 2	NLB	24	1 (4%)

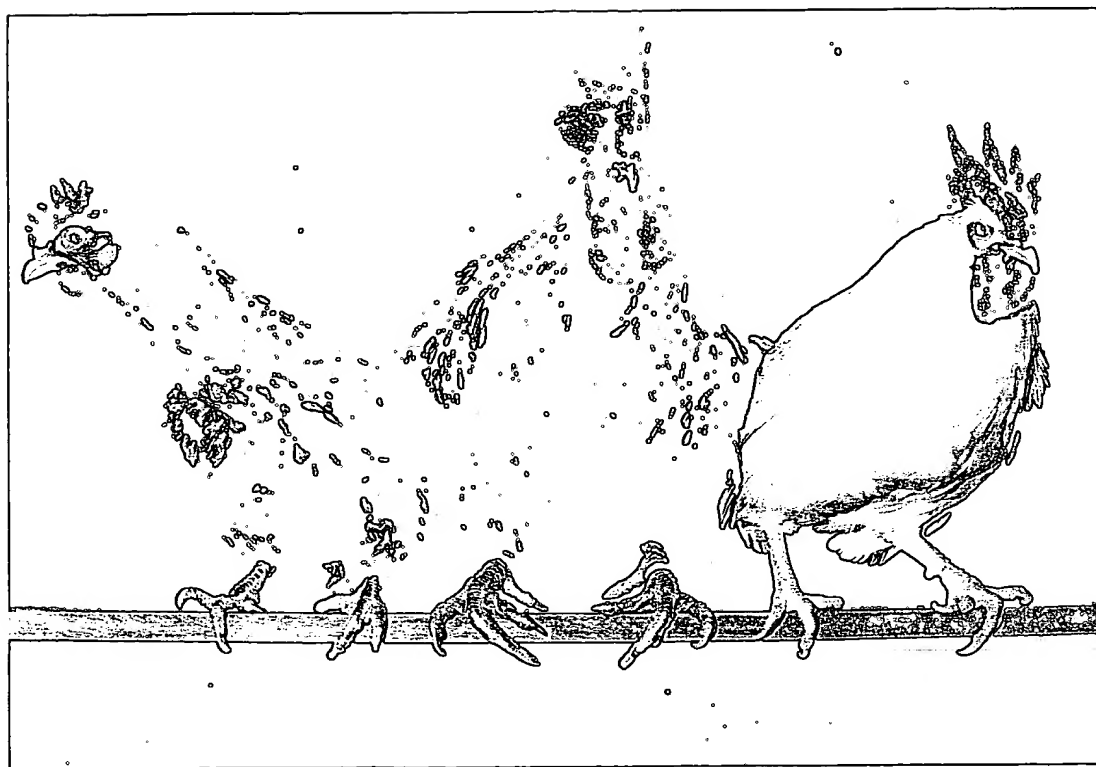


Figure 3. The chimaeric cock 2 (centre) which contained some White Leghorn PGCs transfected with the NLB defective retrovirus. Most of this bird's offspring appeared like normal Rhode Island Red birds (left) but he also produced some offspring (right) with a White Leghorn phenotype ( $F_1$  bird 24) that were transgenic. The Southern blots in figure 2 are from these birds.

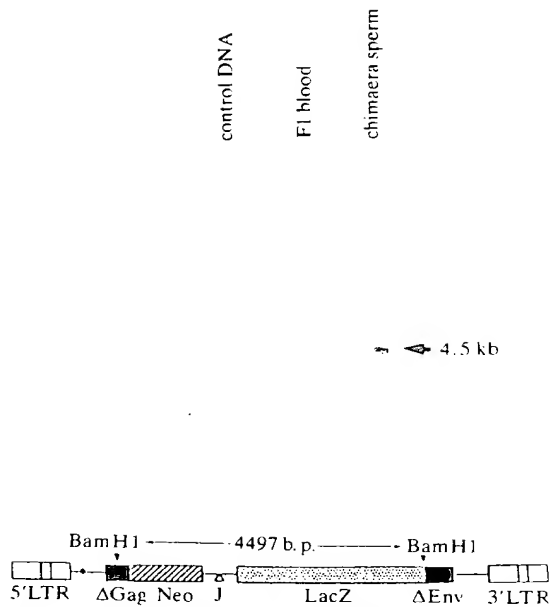


Figure 2. Southern plots of the DNA from the sperm of the chimaeric bird (right, cock 2) and the blood of one of his offspring (centre, F1 bird 24) which is transgenic. The band at 4.5 kb corresponds with the *Bam* H1 fragment of the NLB vector shown in map form at the bottom. Both the sperm of the chimaera and the transgenic offspring contain this foreign DNA which is absent from the DNA of control birds (left).

found in roughly 3% of the embryos injected with pGC-containing blood samples and in 23% of the embryos injected with germinal crescent samples (table 2). Hatchlings were produced from similarly manipulated embryos and five cockerels were raised to sexual maturity. Not all of these produced good samples of sperm for artificial insemination but two cockerels produced  $2-3 \times 10^8$  sperm  $\text{cm}^{-2}$ , and the sperm contained vector DNA. These were therefore selected for breeding experiments. In the case of cock 1, 56 hatchlings were produced and their blood screened for vector DNA before a positive specimen was obtained. With cock 2, which contained NLB-treated pGCs, the 24th chick to be produced was transgenic (table 3). In this latter case the Rhode Island Red (RIR) chimaeric cock was mated with a White Leghorn (WL) hen. This produced two types of offspring. The most common were initially white with black spots; these chicks turned brown in about 3 months. These are the characteristic offspring of a RIR  $\times$  WL cross in our flock. Occasionally, however, a pure White Leghorn was produced (figure 3) which was also shown to be a transgenic bird (figure 2).

#### 4. DISCUSSION

The aim of this work was to demonstrate the feasibility of using primordial germ cells as a means of gaining access into the avian genome. Foreign DNA was introduced into these cells by using two defective retroviruses as these systems have a high efficiency for

infecting cells with single copies of the foreign DNA (Varmus 1988). Two sources of pGCs were used representing cells at different stages of their normal migratory activity.

Primordial germ cells that are obtained from embryonic blood are already in transit to the germinal ridge but they produced chimaeric embryos in only 3% of these experiments. This is probably because the migration of these cells occurs as a short temporal pulse (Al-Thani & Simkiss 1991) that may be missed in individual donor embryos. A much higher rate of chimaera production (23%) was obtained by extracting the primordial germ cells from the germinal crescent before their vascular migration (table 2). Clearly it is the number of primordial germ cells that can be obtained rather than their 'cellular maturity' that determines the success rate in forming these embryos. Chimaeric hatchlings were produced, however, from both blood-derived and germinal crescent-derived pGCs.

The initial experiments used blood-derived pGCs transfected with vSNO21 and injected into line zero embryos. Four cockerels were hatched and raised to sexual maturity from these experiments, and one of them (cock 1) was subsequently used for breeding experiments. Because this cock was a chimaera, those offspring that were derived from its transfected cells should be transgenics, whereas those derived from its own cells would be normal. The production of one transgenic in 56 hatchlings is roughly in keeping with these expectations. The blood source that was used to form this chimaera contained roughly 1-2 pGCs per microlitre before dilution with the vector; the injection volume was roughly 5  $\mu\text{l}$ . Thus 3-5 pGCs would have been injected into a recipient embryo with 200-300 endogenous pGCs. Assuming a high rate of transfection and that the chances of colonizing the germinal ridge are equal for injected and endogenous pGCs, this would produce transgenics in approximately 1-4% of cases, i.e. in the range found. Despite this it was felt that the instability of the defective retrovirus vSNO21 and the absence of an accurate map for this construct made these results inconclusive.

A second series of experiments was therefore done using pGCs from the germinal crescent of a line zero White Leghorn embryo which were transfected with the vector NLB and then injected into a Rhode Island Red recipient. A cockerel produced from this experiment was raised to maturity. Semen from this animal (cock 2), gave a 4.5 kilobase (kb) fragment on Southern blots of *Bam* H1 cut DNA, showing that it contained transfected sperm (figure 2). Crossing this bird with a White Leghorn hen produced two types of offspring. The most common were the characteristic offspring of a cross between a White Leghorn and a Rhode Island Red, but occasionally a pure White Leghorn phenotype was obtained. These were clearly the offspring of sperm derived from the White Leghorn pGCs that had been injected into the Rhode Island Red embryo. The DNA from a blood sample from this white F1 cockerel was analysed and shown to contain the 4.5 kb fragment of vector DNA so that both traditional genetics and DNA screening identified this

bird as being a transgenic derived from the transfected donor PGCs that were used to form the chimaeric cockerel.

These experiments demonstrate, therefore, that it is possible to obtain transfected PGCs that can be used to produce chimaeric birds. The foreign DNA will only be present in some of the germ cells from these birds, but by using PGCs from different strains it is possible to identify such transgenic birds easily. Clearly, the next stage in the improvement of this approach to producing transgenic birds is to increase the efficiency of the procedure, either by sterilizing the recipient embryos (Aige-Gil & Simkiss 1991) or by increasing the input of transformed PGCs. It will be apparent, however, that studying the chimaeric birds that are used in this approach will also provide additional information on their reproductive biology. By measuring the number of copies of vector DNA in the sperm of chimaeric birds it will be possible to quantify the ratio of transfected to normal sperm and thus the probability of producing transgenic offspring. Furthermore, because retroviruses insert into their host DNA in a largely random fashion, it is also possible to cut the vector DNA so as to release a variety of junction fragments that identify each of the individual PGCs that have settled in the gonad. Thus information on both the number of PGCs and their relative fecundity can be obtained from these experiments. At the present time it appears that the number of sperm carrying foreign DNA varies between ejaculates, suggesting that particular regions of the testis may be intermittently involved: this is currently being investigated.

The technique of using PGCs to produce gonadal chimacras and transgenic offspring may also have other implications. There have recently been a number of suggestions that PGCs may be capable of producing embryonic stem (ES) cells in culture (Resnick *et al.* 1992; Matsui *et al.* 1992). If these results can be extended to *in vivo* studies they may provide important techniques for studying the whole process of embryonic differentiation.

We thank Dr N. Bumstead of AFRC Institute of Animal Health and Dr G. Verdier, Dr V.-G. Nigon, Dr Y. Chebloune and Dr C. Legras of Laboratoire de Biologie Cellulaire, Université Claude Bernard for providing us with defective retroviruses. This work was supported by AFRC grants (AT45/521, AT45/600), by Merck Research Laboratories and by an Anglo-French Alliance award (PN92.214).

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